DESCRIPTION

GENE ENCODING PROTEIN FROM MEROZOITE OF *BABESIA CABALLI*, RECOMBINANT PROTEIN OBTAINED WITH SAID GENE AND USE THEREOF

TECHNICAL FIELD

The present invention relates to a protein derived from a merozoite of *Babesia caballi* (hereinafter also referred to as "BC"), a kind of equine Protozoa *Babesia*, a gene encoding said protein, an antibody specific to said protein, and a method for diagnosing equine babesiasis using the same.

BACKGROUND ART

Equine babesiasis is protozoiasis carried by the mites. The pathogen of this disease is equine Protozoa *Babesia*, among which two species of *Babesia caballi* and *Babesia equi* (hereinafter also referred to as "BE") are known.

Equine babesiasis is widely spread all over the world including South Europe, Asia, Russia, the Middle and Near East, Africa, and Central and South America. Clinically, this disease has main symptoms of anemia and jaundice with high fever and progresses either acutely or chronically. In acute cases, its lethality reaches about 10% or even as high as 50% in rare cases although it may somewhat vary with either of the two pathogens. On the other hand, the conditions after prognosis vary with either

of the pathogens and after alleviation the protozoa disappears from peripheral blood but, in case of BE, it is known that horses suffering from this disease remain lifelong BE carriers.

With increase in international trade of horses in recent years, there is a concern about possible spreading of this disease towards "clean" countries such as North America, Australia and the Far East including Japan. Thus, it becomes most important to detect horses infected with this disease at earlier stage. Horses when confirmed infection of this disease are to be sacrificed in order to prevent the disease from spreading. However, in case of BC infection, the protozoa disappears after alleviation and hence it is sufficient to segregate BC-infected horses without need of sacrifice. Also, therapies needed for the disease are different depending on which of the two species of the pathogen protozoa is involved. Therefore, it is of great interest to diagnose which species of the two *Babesia* protozoa infected horses, especially in case of expensive racing horses.

Life cycle of equine Protozoa *Babesia* is similar to that of malaria protozoa. That is, sporozoite that entered into blood stream of a host immediately invades within erythrocytes to become merozoite, which then propagates by division (schizont) within erythrocytes.

Upon collapse of erythrocytes, merozoite is released and infects to other erythrocytes. Erythrocytes with merozoite residing therein are then introduced within the living body of the carrier tick through sucking of blood. In the intestinal tract of tick, certain individuals of merozoite become gametocytes to form sexual gametes. The thus produced male and female gametes are then united together to form zygote which then invades into within the intestinal cells of tick. Via sporokinete, zygote further propagate within various organs of tick and ultimately reach the salivary gland where a large number of sporozoite are produced, leading to further infection.

Usually, equine babesiasis infection is diagnosed by detecting merozoite present in equine blood or antibodies elicited thereto among the life cycle of equine protozoa *Babesia*.

At present, the complement fixation reaction (hereinafter also referred to as "CF") or the indirect fluorescent antibody technique (hereinafter also referred to as "IFA") have primarily been employed for diagnosing equine babesiasis infection. However, due to their low sensitivity in detection, there is the possibility that infection at very early stage or carrier horses fail to be detected. Moreover, in these serological diagnostics, problems sometimes arise in relation to specificity.

Furthermore, since these diagnostics utilize as an antigen the protozoa isolated from blood of horses infected with the protozoa, cost for preparing an antigen and fluctuations in its quality are another problems. Especially in case of BC, an antigen is scarcely available because infected horses are likely to die with severe symptoms of fever and anemia even at early stage when propagation of protozoa is still in low level. This hampers the establishment of stable diagnostics.

In recent years, as an alternative to CF or IFA, Western blot [Int. J. Parasitol. 22(5): 627-630 (1992)], ELISA [Vet. Parasitol. 20: 43-48 (1986); Int. J. Parasitol. 24(3): 341-346 (1994); Vet. Parasitol. 68: 11-26 (1997)], and an approach with DNA probe [Parasitology 102: 357-365 (1991); Vet. Parasitol. 73: 53-63 (1997)] have been reported. However, even these techniques are disadvantageous; Western blot has insufficient sensitivity in detection, ELISA is not so specific that enables distinction between BE and BC and also has a problem in association with availability of an antigen, and the approach with DNA probe requires special instruments such as autoradiography. Therefore, further improvements are needed for diagnostics of equine babesiasis infection under the current situations.

DISCLOSURE OF INVENTION

Under the circumstances, the present inventors investigated the genetic recombination techniques in order to develop a method enabling production of sporozoite antigen of BC in a large amount, and as a result, successfully isolated and purified a gene encoding a desired BC protein useful for that purpose. Using this gene, it is possible to produce the sporozoite protein of BC in a large amount with the recombinant DNA technique.

That is, the present invention provides a gene encoding a protein from merozoite of *Babesia caballi*, a recombinant protein of *Babesia caballi*, an antibody capable of specifically binding to a 48kDa protein of rhoptry, a kind of extrusome, of *Babesia caballi* merozoite, a method for diagnosing equine babesiasis which comprises specifically detecting anti-*Babesia caballi* antibody in equine blood using said recombinant protein as an antigen, and a method for diagnosing equine babesiasis which comprises detecting the presence of merozoite of *Babesia caballi* in equine blood using said antibody.

The present invention, in one aspect, relates to a gene encoding a 48kDa protein of rhoptry of *Babesia caballi* merozoite. The gene according to the present invention encodes a protein having the amino acid sequence shown in SEQ ID NO: 2, or encodes a protein that has the amino acid sequence shown in SEQ ID NO: 2 with one to

several amino acid residues therein being deleted, substituted or added and that is immunologically reactive with an antibody or antiserum elicited by a 48kDa protein of rhoptry of BC merozoite.

The gene of the present invention has preferably the nucleotide sequence shown in SEQ ID NO: 1. Also, the gene of the present invention has a nucleotide sequence that hybridizes to a complementary sequence to the nucleotide sequence shown in SEQ ID NO: 1 and encodes a protein that is immunologically reactive with an antibody or antiserum elicited by a 48kDa protein of rhoptry of BC merozoite.

The gene and fragments thereof according to the present invention are also suitably used for diagnosis of equine babesiasis with procedures such as DNA probe technique or PCR.

The present invention, in the second aspect, relates to a recombinant protein of *Babesia caballi*. The recombinant protein of the present invention has preferably the amino acid sequence shown in SEQ ID NO: 2. The recombinant protein of the present invention also has the amino acid sequence shown in SEQ ID NO: 2 with one to several amino acid residues therein being deleted, substituted or added and is immunologically reactive with an antibody or antiserum elicited by a 48kDa protein of

rhoptry of BC merozoite.

The recombinant protein of the present invention may be expressed, for instance, from a host transformed with a DNA vector into which cDNA having the nucleotide sequence encoding the amino acid sequence shown in SEQ ID NO: 2 is incorporated. The recombinant protein of the present invention may also be expressed from lysogenic bacteria with recombinant phage prepared by infecting *E. coli* with phage into which cDNA having the nucleotide sequence encoding the amino acid sequence shown in SEQ ID NO: 2 is incorporated.

The present invention, in the third aspect, relates to an antibody capable of specifically binding to a 48kDa protein of rhoptry of *Babesia caballi* merozoite. The 48kDa protein of rhoptry of *Babesia caballi* merozoite to which the antibody of the present invention binds may be one naturally occurring or prepared by the recombinant technique. The antibody of the present invention is preferably a monoclonal antibody. The monoclonal antibody of the present invention includes BC11D and BC233D as described hereinbelow.

The present invention, in the fourth aspect, relates to an antigen comprising the recombinant protein of *Babesia caballi* merozoite. The antigen may be used for specifically detecting anti-*Babesia caballi* antibodies

present in equine blood for enabling diagnosis of equine babesiasis. Thus, the present invention, in the fifth aspect, relates to a method for diagnosing equine babesiasis which comprises specifically detecting anti-*Babesia caballi* antibodies present in equine blood by using said recombinant protein as an antigen.

The present invention, in the sixth aspect, relates to a method for diagnosing equine babesiasis which comprises specifically detecting the presence of *Babesia caballi* merozoite in equine blood by using the antibody according the present invention.

A method for diagnosing equine babesiasis may be performed with ELISA, immunochromatography, agglutination, etc.

Patents, publications and literatures cited therein are all incorporated herein for reference.

BRIEF DESCRIPTION OF DRAWINGS

Fig. 1 is a photograph of confocal laser microscopic image showing reactivity of the monoclonal antibody BC11D of the present invention with *Babesia caballi*.

Fig. 2 schematically illustrates construction of pGEX/BC48 wherein cDNA clone BC48 is incorporated that has the nucleotide sequence shown in SEQ ID NO: 1 and encodes a 48kDa protein of rhoptry of BC merozoite.

Fig. 3 is a photograph indicating Western blot analysis that shows reactivity between proteins expressed from lysogenic bacteria of phage clone BC48 and the monoclonal antibody BC11D recognizing the BC merozoite 48kDa protein.

BEST MODE FOR CARRYING OUT THE INVENTION

The gene of the present invention encoding a 48kDa protein of rhoptry of BC merozoite may be obtained, for instance, as described hereinbelow. That is, BC-infected erythrocytes with about 10% of a rate of parasite within erythrocytes are prepared by *in vitro* culture as described by Avarzed et al. [J. Vet. Med. Sci. 59(6), 479-481 (1997)]. Total RNAs are then extracted by guanidinium-phenol-chloroform procedure as described by Chomczynski et al. [Anal. Biochem. 162, 156-159 (1987)]. mRNAs are isolated and purified with oligotex-dT 30 (manufactured by Takara K.K.) and cDNAs are synthesized with the mRNAs using Zap-cDNA synthesizer kit (manufactured by Stratagene Inc.). The obtained cDNAs are inserted into λZap II phage vector (manufactured by Stratagene Inc.), packaged with Gigapack III packaging system (manufactured by Stratagene Inc.) to construct a cDNA library. The obtained cDNA library is screened immunologically using monoclonal antibody recognizing the 48kDa protein of BC merozoite to give a desired cDNA clone, which is recovered as pBluescript clone

by *in vivo* excision.

The cDNA insert of the thus obtained clone is determined for its nucleotide sequence by, for instance, the dideoxy method by Sanger et al. [Proc. Natl. Acad. Sci. USA, 74, 5463 (1977)]. The nucleotide sequence of the cDNA is consisted of 1,828 base pairs in full length as shown in SEQ ID NO: 1 and contains a structural gene of 1,374 base pairs in full length corresponding to the amino acid sequence of a 48kDa protein of BC merozoite as shown in SEQ ID NO: 1 or 2. The thus obtained cDNA directly or after being modified at its 5' end is inserted into the known expression vector at downstream of promoter by the conventional procedure. The expression vector with the inserted cDNA is then introduced into known cells such as *E. coli*, yeast, animal cells or insect cells by the conventional procedure.

INDUSTRIAL APPLICABILITY

In accordance with the present invention, it is possible to produce stably a 48kDa protein of rhoptry of *Babesia caballi* merozoite as well as a gene encoding said protein in a large amount by the recombinant DNA technique. A protein obtained from the gene of the present invention or from cells wherein said gene is introduced, or a polypeptide constituting a portion of said protein, may be used as an antigen for detecting anti-merozoite antibodies

of BC present in equine blood for use in diagnosis of equine babesiasis. Such a protein and a polypeptide constituting a portion of said protein may also be used as an antigen for preparing anti-BC merozoite antibodies, especially a monoclonal antibody to BC merozoite. The anti-BC merozoite antibody thus prepared may be used for detecting BC merozoite in equine blood for use in diagnosis of equine babesiasis.

EXAMPLE

The present invention is explained in more detail by means of the following Examples but it should not be construed to be limited thereto.

Example 1: Construction of cDNA Library of *Babesia caballi* Merozoite

BC-infected erythrocytes with about 10% of a rate of parasite within erythrocytes were prepared by *in vitro* culture as described by Avarzed et al. [J. Vet. Med. Sci. 59(6), 479-481 (1997)]. That is, blood was drawn from horses infected with BC (USDA strain) into a tube charged with EDTA as a coagulating agent. The tube was centrifuged with RPMI1640 medium supplemented with 10 mM HEPES and washed and buffy coat was removed. After centrifugation and washing, a supernatant was discarded and sediment 50 µl was mixed with 1 ml of RPMI1640 medium (containing 2 mM L-glutamine and 50 µl normal equine erythrocyte) supplemented

with 40% equine serum. The mixture was added to a 24-well microtiter plate at 1 ml/well. The microtiter plate was incubated at 37°C with conditions of 5% CO_2, 2% O_2 and 93% N_2. While incubation, the culture medium was replaced with fresh medium and a rate of parasite was measured by the Giemsa staining everyday with passage being performed whenever appropriate.

From the thus obtained BC-infected erythrocytes, cDNA library was constructed as reported by Ikadai et al. [The 126th Japan Veterinary Association, excerpt, page 191 (1998)]. That is, total RNAs were extracted from the BC-infected erythrocytes by the guanidinium-phenol-chloroform method as described by Chomczynski et al. [Anal. Biochem. 162, 156-159 (1987)]. mRNAs were isolated and purified from the total RNAs with oligotex-dT 30 (manufactured by Takara K.K.) and then cDNAs were synthesized with Zap-cDNA synthesizer kit (manufactured by Stratagene Inc.) in accordance with the protocol attached thereto. The cDNAs were inserted into λZap II phage vector (manufactured by Stratagene Inc.) and packaged with Gigapack III packaging system (manufactured by Stratagene Inc.) in accordance with the protocol attached thereto to construct cDNA library.

Example 2: Production of Monoclonal Antibody Recognizing 48kDa Antigen of *Babesia caballi* Merozoite

As an antigen, a suspension of 1×10^8 merozoite

from BC-infected horses in 0.1 ml phosphate buffer was emulsified with Freund's complete adjuvant (manufactured by Difco). The emulsion (0.2 ml/mouse) was inoculated intraperitoneally and subcutaneously to BALB/c mice of 7 weeks old. A suspension of the same amount of merozoite with Freund's incomplete adjuvant (manufactured by Difco) was boostered three times with intervals of two weeks. Three days after the fourth immunization, merozoite was administered intravenously to mice. Three days later mice were dissected and the spleen was removed. The spleen cells were fused with Sp-2 mouse myeloma cells with polyethylene glycol (PEG 1500, manufactured by Boehringer Mannheim Biochemica).

The hybridoma cells were selected with HAT medium (manufactured by Boehringer Mannheim Biochemica) and GIT medium (manufactured by Wako K.K.) supplemented with Bri Clone (manufactured by BioResearch) in conventional manner. The hybridoma cells were screened for their supernatant by the indirect fluorescent antibody procedure with smear of BC-infected erythrocytes fixed with cold acetone to thereby give six clones. Among these, monoclonal antibodies produced by two hybridomas, referred to as "BC11D" and "BC233", were found to recognize the same 48kDa antigen by Western blot with solubilized antigen of BC merozoite. It was confirmed that neither of the monoclonal antibodies

produced by BC11D and BC233D reacted with BE and non-infected equine erythrocytes by Western blot. These monoclonal antibodies recognizing the 48kDa antigen were found to recognize rhoptry by observation with confocal laser microscope (Fig. 1). Subclass and type of L chain of the monoclonal antibodies produced by these hybridoma clones were determined to be IgG2a and IgG1, respectively, with Amersham isotyping kit (manufactured by Amersham).

Example 3: Screening of cDNA Library of BC Merozoite and Sequencing of cDNA Clone

For a primary antibody, culture supernatant of the monoclonal antibody produced by BC11D recognizing the 48kDa protein prepared in Example 2 was diluted 5-folds with PBS supplemented with 1% bovine serum albumin. As a secondary antibody capable of binding to the primary antibody was used alkali phosphatase-conjugated goat anti-mouse IgG antibody (manufactured by Jackson Immunoresearch Laboratories, Inc.) diluted 20,000-folds with PBS supplemented with 1% bovine serum albumin. The cDNA library obtained in Example 1 was screened immunologically with the primary and secondary antibodies. Positive plaque was recovered and cloned. The obtained cDNA clone BC48 was inserted into pBluescript SK (+) plasmid vector (manufactured by Stratagene Inc.) by *in vivo* excision. Thereafter, the cDNA was cleaved out of the vector with

restriction enzymes and subcloned. The inserted DNA was determined for its nucleotide sequence by the dye primer method using M13 reverse and universal primers (manufactured by Stratagene Inc.) with ABI PRSMTM 377 sequencer (manufactured by Perkin Elmer). The obtained sequence data were analyzed with Gene Works (manufactured by IntelliGenetics, Inc.). As a result, it was found that the gene encoding the 48kDa antigen of BC merozoite had the nucleotide sequence shown in SEQ ID NO: 1 of 1,828 base pairs in full length. The gene was found to contain 1,374 base pairs in full length for a structural gene that encodes the amino acid sequence of the 48kDa protein of BC merozoite as shown in SEQ ID NO: 1.

A plasmid vector pGEX/BC48, i.e. pGEX4T-3 wherein the cDNA clone BC48 was incorporated, after transfection into *E. coli*, has been deposited as Escherichia coli/GST-BC48 at the Fermentation Research Institute Agency of Industrial Science and Technology, 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, 305-8566, Japan with accession number: FERM BP-6761 on June 16, 1999.

Example 4: Preparation of Recombinant BC Merozoite

The cDNA inserted in pBluescript SK (+) plasmid vector was then cleaved with EcoRI and XhoI and inserted into pGEX4T-3 plasmid vector (manufactured by Pharmacia Biochemicals Inc.) at the EcoRI and XhoI sites (Fig. 2).

The obtained plasmid vector was transfected into *E. coli* (BL21 strain) and expression was induced with isopropyl-β-D-thio-galactopyranoside (IPTG).

After expression, the suspension (500 ml) of *E. coli* was centrifuged at 6,000 rpm at 4°C for 10 minutes and supernatant was discarded. The sediment was suspended in a sonication buffer (50 mM Tris-HCl (pH 8.0)/50 mM NaCl/1 ml EDTA; 10 ml) and sonicated to rupture cells. To the suspension of ruptured cells was added 10% Triton X-100 at a final concentration of 1%. The mixture was centrifuged at 12,000 rpm at 4°C for 30 minutes and supernatant was recovered. To the supernatant was added 0.2 ml of 50% slurry of Glutathione sepharose 4B beads (manufactured by Pharmacia Biochemicals Inc.) and mixed at 4°C for 30 minutes. The mixture was centrifuged at 3,000 rpm at 4°C for 10 minutes and supernatant was discarded. The sediment was mixed with PBS supplemented with 1 ml of 0.5% Triton X-100 (PBST). The mixture was centrifuged at 5,000 rpm at 4°C for 10 seconds and supernatant was discarded. These procedures were repeated twice and washed. Thereto was added a buffer for suspending thrombin (50 mM Tris-HCl (pH 8.0)/150 mM NaCl/2.5 mM $CaCl_2$; 1 ml) and mixed. The mixture was centrifuged at 5,000 rpm at 4°C for 10 seconds and supernatant was discarded. To the sediment was added 0.5 ml of a dispersion buffer containing thrombin at a

final concentration 20 U and mixed at 4°C overnight. The mixture was centrifuged at 3,000 rpm at 4°C for 10 minutes and recovered supernatant was used as the recombinant protein [cf. Schelp et al., Appl. Parasitol. 36, 1-10 (1995)].

The obtained recombinant protein was blotted onto nitrocellulose membrane (HybondTM-C extra, Amersham). Western blotting was performed using the monoclonal antibody produced by BC11D prepared in Example 2 as a primary antibody and peroxidase-conjugated goat anti-mouse IgG antibody (manufactured by Jackson Immunoresearch Laboratories, Inc.) capable of binding to the primary antibody as a secondary antibody. As a result, it was found that the recombinant protein reacted with the monoclonal antibody produced by BC11D prepared in Example 2 and a molecular weight of the expressed protein corresponded to the 48kDa protein derived from BC protozoa (Fig. 3).

Example 5: Analysis for Distinction Between BC and BE by ELISA Using Recombinant Antigen

ELISA was performed as reported by Takumi et al. [Jpn. J. Vet. Sci. 52(2), 241-250 (1990)]. That is, the expressed protein obtained in Example 4 was diluted with 0.05 M carbonated/bicarbonate buffer (pH 9.6) and added to 96-well plate for ELISA at 50 µl/well and incubated at 4°C

overnight to immobilize the protein. After immobilization, the plate was washed once with PBS supplemented with 0.05% Tween 20 and to the plate was added PBS supplemented with 3% skimmed milk at 100 µl/well. The plate was incubated at 37°C for 60 minutes for blocking. After blocking, the plate was washed once with PBS supplemented with 0.05% Tween 20. To the plate was added samples diluted to 1/80 with PBS supplemented with 3% skimmed milk at 50 µl/well and the plate was incubated at 37°C for 60 minutes. The samples used were serum from horses experimentally infected with either BC or BE and equine serum infected with neither of BC nor BE prepared in the Racing Horse Comprehensive Laboratory, Japan Racing Association. After completion of reaction, the plate was washed six times with PBS supplemented with 0.05% Tween 20 and to the plate was added peroxidase-conjugated anti-horse IgG antibody (manufactured by Cappel) diluted to 1/4,000 with PBS supplemented with 3% skimmed milk at 50 µl/well. The plate was incubated at 37°C for 60 minutes. After completion of reaction, the plate was washed six times with PBS supplemented with 0.05% Tween 20. To the plate was added a solution of 0.1M citric acid, 0.2M sodium phosphate, 0.003% hydrogen peroxide and 0.3 mg/ml 2,2'-azino-bis(3-ethylbenzothizolin-6-sulphonic acid)(manufactured by Sigma) at 100 µl/well. The plate was incubated at room temperature for 60 minutes and thereafter

absorbance at 415 nm was measured for each well. The results are shown in Table 1.

Table 1

ELISA Value of Equine Serum Infected with Neither of BC nor BE	ELISA Value of Equine Serum Experimentally Infected with BE	ELISA Value of Equine Serum Experimentally Infected with BC
0.039	0.018	0.319
0.021	0.032	0.541
0.003	0.045	0.805
0.014	0.033	0.700
0.029		0.721
0.020		
0.068		
0.017		

ELISA was performed with BC-negative equine serum to reveal that the ELISA had positive limitation of 0.2. As a result of ELISA using the recombinant antigen, ELISA value for equine serum infected neither with BC nor BE and for equine serum infected with BE was not more than 0.2 whereas it was 0.319 to 0.805 for equine serum infected with BC, indicating difference in specificity.